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Engineering PQQ glucose dehydrogenase with improved substrate specificity Site-directed mutagenesis studies on the active center of PQQ glucose dehydrogenase

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Abstract

Site-directed mutagenesis was carried out on the active site of water-soluble PQQ glucose dehydrogenase (PQQGDH-B) to improve its substrate specificity. Amino acid substitution of His168 resulted in a drastic decrease in the enzyme's catalytic activity, consistent with its putative catalytic role. Substitutions were also carried out in neighboring residues, Lys166, Asp167, and Gln169, in an attempt to alter the enzyme's substrate binding site. Lys166 and Gln169 mutants showed only minor changes in substrate specificity profiles. In sharp contrast, mutants of Asp167 showed considerably altered specificity profiles. Of the numerous Asp167 mutants characterized, Asp167Glu showed the best substrate specificity profile, while retaining most of its catalytic activity for glucose and stability. We also investigated the cumulative effect of combining the Asp167Glu substitution with the previously reported Asn452Thr mutation. Interpretation of the effect of the replacement of Asp167 to Glu on the alteration of substrate specificity in relation with the predicted 3D model of PQQGDH-B is also discussed.

Keywords: Pyrroloquinoline quinone (PQQ); Glucose dehydrogenase (GDH); Substrate specificity; Protein engineering

1. Introduction

Pyrroloquinoline quinone (PQQ)-harboring water-soluble glucose dehydrogenase (PQQGDH-B or sGDH) has great potential as a constituent of electron mediator-type glucose sensors because it does not utilize dissolved oxygen as electron acceptor and has high catalytic activity (over 5000 U mg^{-1} protein versus 300 U mg^{-1} protein for glucose oxidase) [1–12]. However, the substrate specificity of PQQGDH-B is broad, catalyzing the oxidation of a number of monosaccharides and disaccharides such as galactose, lactose, and maltose that might become the impurities toward blood glucose monitoring. The narrowing of the substrate specificity for glucose is therefore greatly desired for future application of PQQGDH-B in glucose sensor development. Moreover, PQQGDH-B can also be utilized for the determination of redox mediators, phenols, and catecholamines in the presence of excess glucose. Detection systems of phenolic compounds have been developed using PQQGDH-B as a sensor constituent [13–17].

The authors have been carrying out protein engineering studies of both membrane-binding PQQGDH (PQQGDH-A) and water-soluble PQQGDH (PQQGDH-B) in order to create ideal enzymes for glucose sensing [12]. Prior to predicted model of PQQGDH-A and information of the tertiary structure of PQQGDH-B [34], we have carried out a series of site-directed mutagenesis studies. Substrate specificity, thermal stability, and cofactor-binding stability have been improved by mutating PQQGDH-A [18–28]. Among the mutants of PQQGDH-B [29–33], Ser231Lys has an eight-fold greater thermal stability at 55 °C [29]

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and Glu277Lys has an improved catalytic efficiency [31]. Attempting to repeat the success in narrowing the substrate specificity of PQQGDH-As [23,28], we focused on the C-terminal region of PQQGDH-B for introducing mutations. The resulting Asn452Thr mutant showed a narrower substrate specificity profile compared with wild-type, with the relative activity toward lactose and maltose being particularly decreased [32]. However, further improvements of substrate specificity remain necessary. We also previously reported that improvement of quaternary structure stability resulted in the increase of thermal stability of this 100 kDa homodimeric enzyme [33]. Using the recently elucidated high-resolution 3-D structure of PQQGDH-B [34–36], we introduced a disulfide bond at the dimer interface, resulting in a greatly improved thermal stability without losing catalytic activity.

The monomeric form of PQQGDH-B has one PQQ cofactor molecule and three Ca^{2+} ions, two of which are located in the dimer interface and the third Ca^{2+} ion is located near PQQ. Each subunit forms a β-propeller fold, a four-stranded anti-parallel β-sheet referred to as "W-motif". The strands of each W-motif are labeled A to D from the inside to the outside of the molecule [37]. According to the structural information of PQQGDH-B, the loop regions are related to functions like substrate binding, cofactor binding, and catalysis. The cleft in the center of the propeller of PQQGDH-B, which may contain the active site, is composed of loops 1D2A, 2D3A, 4BC, 4D5A, and 6BC [35]. PQQ is surrounded by Arg252, Asn253 (loop3D4A), Gly271, Pro272 (loop4BC), Thr372 (lop4D5A), Lys401 (loop5BC), Arg430, Arg432 (loop5D6A) and Asp448 (loop6BC). Gln100 (loop1D2A), Asp167, His168 (loop2D3A), Gln192, Leu193 (loop3BC), Arg252 (loop3D4A) and Tyr367, Trp370 (loop4D5A) are located near the substrate. Based on the 3-D structure, the loop 2D3A region was proposed by Oubrie et al. [34] to be important for substrate recognition, with His168 having a crucial role in the oxidation of glucose [36]. However, there is no experimental evidence supporting this proposal.

In this paper, we investigate the impact of amino acid substitutions within the loop2D3A region, containing the putative active site His168. Based on our results, we created the Asp167Glu/Asn452Thr mutant, which shows improved specificity for glucose.

2. Materials and methods

2.1. Site-directed mutagenesis

The PQQGDH-B expression vector pGB [29] was constructed by amplifying a PQQGDH-B structural gene and inserting it into pTrc99A (Pharmacia, Sweden). Site-directed mutagenesis was carried out using the Mutan-Express K_m kit (Takara, Kyoto, Japan), according to the manufacturer's instructions, after transferring a 1.2 kbp *Kpn*I–*Hin*dIII fragment containing the gene from pGB to pKF18k. The sequences of the oligonucleotides used for the mutagenesis are summarized in Fig. 1. The mutations were confirmed using the automated DNA sequencer ABI PRISM Genetic analyzer 310 (Applied Biosystems, California, US). The mutated genes were digested with *Kpn*I and *Hin*dIII and replaced into pGB to construct expression vectors containing mutated PQQGDH-B.

2.2. Determination of kinetic parameters of PQQGDH-Bs

Enzyme was prepared as previously described [29,31]. GDH activity was measured using 0.6 M phenazine methosulfate (PMS) and 0.06 mM 2,6-dichlorophenolindophenol (DCIP) after incubation for 30 min in 10 mM MOPS–NaOH (pH 7.0) containing 1 μM PQQ and 1 mM $CaCl_2$. The activity was determined by measuring the decrease in absorbance of DCIP at 600 nM. The substrate specificity profiles of the enzymes were determined using the following nine different substrates: glucose, 2-deoxy-glucose, mannose, allose, 3-*O*-methyl-glucose, galactose, xylose, lactose, and maltose.

The thermal stability of the wild-type and mutant enzymes was determined using 0.3 μg/ml protein as previously described [29]. Since the initial time course for thermal inactivation at 55 °C followed first-order kinetics, the thermal stability of each mutant enzyme was expressed as a half-life. Following the 30 min preincubation described above, each

		171	170	169	168	167	166	165	164	163	
		Gly	Ser	Gln	His	Asp	Lys	Ser	Ser	Pro	
Wild type	5'- CG	ACC	TGA	CTG	ATG	GTC	TTT	TGA	TGA	AGG	-3'
Asp167Ala	5'-	CC	TGA	CTG	ATG	GGC	TTT	TGA	TGA	AGG	-3'
Asp167Cys	5'-	CC	TGA	CTG	ATG	GCA	TTT	TGA	TGA	AGG	-3'
Asp167Glu	5'-	CC	TGA	CTG	ATG	TTC	TTT	TGA	TGA	AGG	-3'
Asp167Gly	5'-	CC	TGA	CTG	ATG	GTG	TTT	TGA	TGA	AGG	-3'
Asp167His	5'- CG	ACC	TGA	CTG	ATG	TTT	TTT	TGA	TGA	AGG	-3'
Asp167Lys	5'-	CC	TGA	CTG	ATG	GTT	TTT	TGA	TGA	AGG	-3'
Asp167Gln	5'- CG	ACC	TGA	CTG	ATG	TTG	TTT	TGA	TGA	AGG	-3'
Asp167Arg	5'-	CC	TGA	CTG	ATG	GCT	TTT	TGA	TGA	AGG	-3'
Asp167Ser	5'-	CC	TGA	CTG	ATG	GCG	TTT	TGA	TGA	AGG	-3'
Asp167Val	5'-	CC	TGA	CTG	ATG	GAC	TTT	TGA	TGA	AGG	-3'
Asp167Trp	5'- CG	ACC	TGA	CTG	ATG	CCA	TTT	TGA	TGA	AGG	-3'
Asp167Tyr	5'-	CC	TGA	CTG	ATG	GTA	TTT	TGA	TGA	AGG	-3'

Fig. 1. The sequences of the oligonucleotide primers used for site-directed mutagenesis of PQQGDH-B. The corresponding amino acid sequence is shown above.

enzyme sample was subjected to thermal inactivation experiments. Thermal inactivation was measured by incubating the holo-enzyme in a total volume of 200 μl of 10 mM MOPS–NaOH (pH 7.0) at 55 °C. Aliquots were taken every 5 min and placed at 4 °C for 2 min followed by incubation at room temperature for 30 min. The residual enzyme activity was determined as described above.

EDTA tolerance was determined by incubating wild-type and mutant PQQGDH-B in 10 mM MOPS–NaOH (pH 7.0) containing 5 mM EDTA and periodically measuring residual activity of the aliquots.

2.3. Calculation of kinetic parameters of PQQGDH-Bs

The enzymatic reaction curve of PQQGDH-B showed a slight deviation from Michaelis–Menten kinetics, showing negative cooperativity [38]. To simplify the determination of Michaelis constants (K_m and V_{max}), the points at low and high glucose concentrations were ignored to produce linear plots.

2.4. Three dimensional structure prediction

Three-dimensional predictions were performed using the molecular operating environment (MOE) (Chemical Computing Group Inc., Quebec, Canada). By using available PDB data [35], 1C9U (oxidized form without glucose) and 1CQ1 (reduced form with glucose), we firstly made the structure of oxidized PQQGDH-B-glucose complex, since the oxidized form was not reported in the complex form. We superimposed two structures and then removed all the 1CQ1 structural data except glucose. Based on this structure, molecular dynamics (MD) calculation was performed from 0 to 1 ns in CHARMM22 as a force field. Then, we extracted the candidates every 100 fs. Among the extracted structures (10000 candidates), we determined the structure having the lowest potential energy as a final structure. In the case of Asp167Glu, same operation was also executed after the replacement of Asp167 to Glu with possible side chain orientation. MD calculation was performed following condition. Firstly, addition of hydrogen atoms to PQQGDH-B structure by MOE and optimization of orientation of some hydrogen atoms. Only side chains of the residues located within 5.0 Å from glucose molecule is a target for MD calculation. Except them, such as main chain, side chains that are 5.0 Å away from glucose, PQQ molecule and Ca^{2+} ions are fixed. With respect to glucose molecule, carbon and oxygen atoms are fixed and hydrogen atoms are unfixed. Water molecules are excluded from MD calculation because there are no molecules mediating hydrogen bond between side chain and glucose.

Table 1
Kinetic parameters of wild-type and His168 mutants for glucose

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Wild-type	25	3860	154
His168Cys	193	2.5	12.9×10^{-3}
His168Gln	154	0.8	5.2×10^{-3}

3. Results

3.1. Site-directed mutagenesis of Lys166, Asp167, His168, and Gln169

We created and characterized two His168 mutants of PQQGDH-B, His168Cys and His168Gln. As shown in Table 1, both mutant enzymes have drastically decreased k_{cat} values (His168Cys: 2.5 s^{-1}, His168Gln: 0.8 s^{-1}) and increased K_m values (His168Cys: 193 mM, His168Gln: 154 mM). The resulting catalytic efficiencies (k_{cat}/K_m) of His168Cys and His168Gln are 12.9×10^{-3} and 5.2×10^{-3} s^{-1} mM^{-1}, respectively. These catalytic efficiency values are 11900- and 29600-fold lower than that of wild-type (154 s^{-1} mM^{-1}). Based on the 3-D structure of PQQGDH [35], His168 was proposed to be positioned in the active center and involved mainly in the affinity and oxidation of the substrate. The greatly decreased catalytic efficiencies resulting from mutation of His168 is consistent with this residue playing a significant role in the oxidation of substrates.

Table 2
Substrate specificities of Lys166 and Gln169 mutants

	Substrate concentration: 100 mM				Substrate concentration: 20 mM		
	Wild-type	Lys166Glu	Lys166Ile	Lys166Gly	Wild-type	Gln169Glu	Gln169Lys
K_m for Glc (mM)	25	44	39	34	25	20	10
Glucose	100 (%)	100 (%)	100 (%)	100 (%)	100 (%)	100 (%)	100 (%)
2-Deoxy-glucose	7	3	9	5	4	4	14
Mannose	10	12	18	12	13	7	0
Allose	66	80	76	61	47	56	91
3-*O*-Methyl-glucose	102	92	97	89	81	87	90
Galactose	10	13	113	10	11	6	18
Xylose	10	13	24	12	7	6	24
Lactose	54	58	71	45	61	48	83
Maltose	58	60	67	44	61	85	130

Table 3
Substrate specificities of Asp167 mutants

	Wild-type	Asp167Gly	Asp167Ala	Asp167Val	Asp167Trp	Asp167Ser	Asp167Asn	Asp167Gln	Asp167Glu	Asp167Cys	Asp167Tyr	Asp167His	Asp167Lys	Asp167Arg
K_m for Glc (mM)	25	50	169	162	130	129	74	28	55	77	134	81	180	182
Specific activity ($U\,mg^{-1}$)	174	1.1	2.7	0.5	1.1	5.6	26	42	159	1.7	0.3	5.1	1.2	0.3
Glucose	100 (%)	100 (%)	100 (%)	100 (%)	100 (%)	100 (%)	100 (%)	100 (%)	100 (%)	100 (%)	100 (%)	100 (%)	100 (%)	100 (%)
2-Deoxy-glucose	7	0	12	147	7	16	0	0	0	5	14	0	3	9
Mannose	10	0	1	27	6	3	0	0	0	1	2	0	15	15
Allose	66	2	51	418	83	78	5	2	2	50	81	36	6	35
3-*O*-Methyl-glucose	102	16	49	92	109	47	33	21	3	38	109	46	2	29
Galactose	10	3	12	15	20	15	8	13	4	19	21	23	15	18
Xylose	10	0	0	5	4	1	0	0	0	1	2	0	1	11
Lactose	54	21	68	53	61	66	62	59	32	57	57	86	54	55
Maltose	58	0	13	36	15	15	14	4	10	22	17	0	16	48

Each enzyme activity was measured at 100 mM substrate concentration and compared with glucose.

Amino acid substitutions were then introduced at Lys166, Gln169, and Asp167 of PQQGDH-B, the neighboring residues of His168. Tables 2 and 3 summarize the enzymatic properties of the created mutants using crude enzyme preparations. Lys166 mutants showed increased K_m values for glucose (Lys166Glu: 44 mM, Lys166Ile: 39 mM, and Lys166Gly: 34 mM). However, these mutants showed similar substrate specificity profiles to wild-type (Table 2) and were therefore not considered to be desirable.

We created 13 Asp167 mutants by substituting the residue to Ala, Cys, Glu, Gly, His, Lys, Asn, Gln, Arg, Ser, Val, Trp, and Tyr. Most Asp167 mutants showed 30- to 580-fold decreased GDH activity, while substitution to Asn, Gln, and Glu produced more modest decreases in activity of 85, 76, and 9%, respectively (Table 3). Substitution of Asp167 also caused two- to seven-fold increases in K_m values for glucose except for Asp167Gln, whose K_m value was unaffected by the mutation. In terms of substrate specificity profiles, Asp167Glu, Asp167Gly, Asp167Asn, and Asp167Gln showed drastically lower reactivity toward allose and 3-*O*-methyl-glucose, resulting in narrower substrate specificities than the wild-type enzyme (Table 3). Especially, Asp167Glu showed 2 and 3% activities with allose and 3-*O*-methyl-glucose, respectively, relative to glucose, compared to the 66 and 102% relative rates of the wild-type enzyme. Furthermore, the relative activities of Asp167Glu for the disaccharides lactose and maltose were decrease to 32 and 10%, respectively, compared to the wild-type relative activities of 54 and 58%, respectively. In contrast, the replacement of Asp167 to Val resulted in a broader substrate specificity profile (Table 3), with the relative activities toward 2-deoxy-glucose and allose increasing to 147 and 418%, respectively. The Asp167Val mutant very efficiently oxidizes 2-deoxy-glucose, which is a very poor substrate for the wild-type enzyme [31,32,39].

3.2. Characterization of Asp167Glu

The Asp167Glu mutant retained high catalytic activity while showing some improvement in substrate specificity profile and was therefore selected for further characterization in the purified form. The enzymatic reaction curves of Asp167Glu and wild-type PQQGDH (Fig. 2a and b) show clear differences in their saturation points. The wild-type enzyme reached saturation (4436 U mg^{-1}) at 50 mM glucose, a concentration at which the mutant enzyme has approximately half of its maximum activity. The Asp167Glu mutant reached saturation (2059 U mg^{-1}) at the much greater concentration of 400 mM glucose, a concentration at which the wild-type enzyme demonstrates considerable substrate inhibition with half its maximum reaction rate. From these curves, K_m values for the wild-type GDH and Asp167Glu for glucose were calculated to be 26 and 55 mM, respectively. The relaxation of substrate inhibition by the mutation therefore appears to result from a decrease in affinity toward glucose. The catalytic efficiency (k_{cat}/K_m) of Asp167Glu for glucose (31 s^{-1} mM^{-1}) is approximately a quarter that of wild-type. K_m values were also increased by the Asp167Glu mutation for other substrates, from 35.5 to 199.0 mM for allose, from 28.7 to 99.0 mM for 3-*O*-methyl-glucose, from 18.9 to 77.0 mM for lactose, from 26.0 to 156.0 mM for maltose and from 14.0 to 17.0 mM for cellobiose (Table 4). The K_m values for galactose were not determined.

The Asp167Glu mutation was also found to result in a five-fold increase in thermal stability, with the wild-type and mutant enzymes having half-lives at 55 °C of 5 and 25 min, respectively (Fig. 3). The presence of bivalent metal ions is essential in all PQQ-harboring enzymes for binding of the PQQ cofactor to the active site. Evaluation of the EDTA tolerance of PQQ enzymes generally reflects the stability of cofactor binding. The EDTA tolerance of the Asp167Glu mutant was found comparable to that of the wild-type enzyme (data not shown).

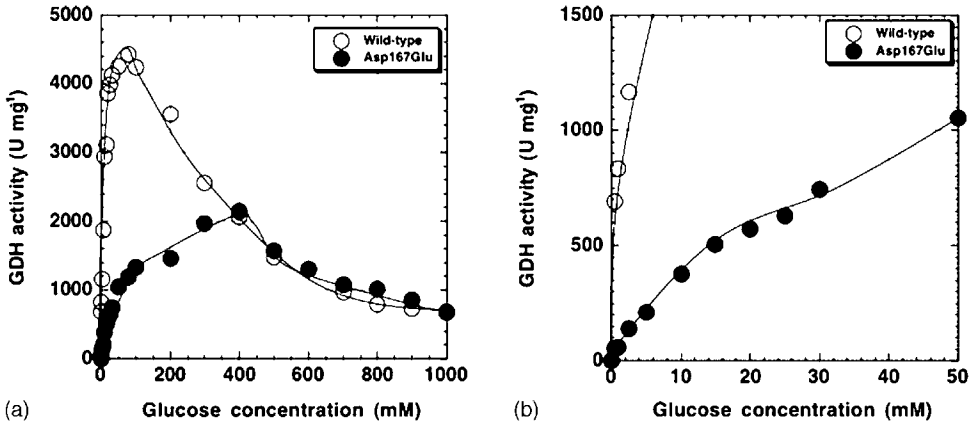

Fig. 2. Enzymatic reaction curves of wild-type (○) and Asp167Glu (●) PQQGDH-Bs with increasing glucose concentration of 0–1000 mM (a) and 0–50 mM (b).

Table 4
Kinetic parameters and substrate specificities of PQQGDH-Bs

	wild-type			Asp167Glu			Asn452Thr			Asp167Glu/Asn452Thr		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Glucose	25.0	3860	154 (100%)	55.0	1724	31 (100%)	12.5	1791	143 (100%)	48.0	1193	25 (100%)
Allose	35.5	2509	71 (46%)	199.0	558	3 (10%)	38.7	949	25 (17%)	182.0	73	0.4 (2%)
3-*O*-Methyl-glucose	28.7	3011	105 (68%)	99.0	541	6 (19%)	27.6	1253	45 (31%)	198.0	215	1.1 (4%)
Galactose	5.3	232	44 (29%)	–	–	–	3.7	72	23 (16%)	145.0	89	0.6 (2%)
Lactose	18.9	1659	88 (57%)	77.0	478	6 (19%)	33.6	1038	31 (22%)	55.0	167	3 (12%)
Maltose	26.0	1930	74 (48%)	156.0	436	3 (10%)	46.5	1002	15 (11%)	147.0	65	0.4 (2%)
Cellobiose	14.0	1355	97 (63%)	17.0	1073	63 (203%)	14.0	1060	76 (53%)	16.0	226	14 (56%)

Substrate specificity of each enzyme is shown by comparing catalytic efficiency (k_{cat}/K_m) toward each substrate. The catalytic efficiency of each enzyme toward glucose is set as 100%. "–": not determined.

3.3. Effects of combining Asp167Glu with Asn452Thr

The Asp167Glu was then combined with the previously reported Asn452Thr mutation [32] to investigate the cumulative effect of the two mutations, which individually confer improved specificities. Comparison of the enzymes' substrate specificities (ratios of k_{cat}/K_m) showed that Asp167Glu/Asn452Thr has a narrower specificity than both single mutations. The k_{cat}/K_m values of Asp167Glu/Asn452Thr for lactose and maltose are 12 and 2%, respectively, of the value for glucose (Table 4). The activities toward allose and 3-*O*-methyl-glucose were comparable to Asp167Glu (data not shown). The catalytic activity of the double mutant was comparable to that of the other PQQGDH-Bs (1193 s^{-1}), with a K_m value for glucose of 48 mM. The cumulative effect of combining the Asp167Glu and Asn452Thr mutations resulted in a GDH with a greatly narrowed substrate specificity, while retaining a large proportion of its original catalytic activity.

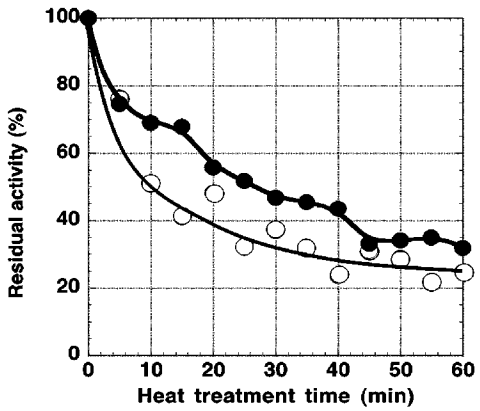

Fig. 3. Thermal stability of wild-type and Asp167Glu PQQGDH-Bs. Aliquots were taken every 5 min to measure residual activity of wild-type (○) and Asp167Glu (●) enzymes (0.3 μg/ml) incubated at 55 °C as described in Section 2.

4. Discussion

In this study, we introduced several amino acid substitutions into the loop2D3A region of PQQGDH-B, where the postulated catalytic residue His168 is located. Because His168 is the only base near the O1 atom of glucose, it is assumed to carry out the initial proton abstraction from the substrate. The drastic decreases in catalytic activity observed with His168Cys and His168Gln, over 4 orders of magnitude lower than wild-type, is consistent with the role of His168 as the catalytic base in PQQGDH-B, previously proposed from the elucidated structural information.

After confirming the catalytic importance of His168, the neighboring residues Lys166, Asp167, and Gln169 were selected for mutagenesis because of their potential interactions with glucose. Although mutants of Lys166 and Gln169 showed altered K_m values, the substrate specificity profiles were not greatly modified. In contrast, the Asp167Gly, Asp167Asn, Asp167Glu, and Asp167Gln mutants showed significant alteration in substrate specificity profiles. Olsthoorn and Duine [39] as well as our group [31,32] have reported that PQQGDH-B can react with the epimer or derivatives of the third hydroxyl group of glucose. According to the structure of the active site of wild-type PQQGDH-B, there are no residues in close proximity to the third hydroxyl group of the glucose substrate [35]. Considering that while the rate of activity of Asp167Glu with glucose was unaffected by the mutation, the drastic reduction in activity with allose and 3-*O*-methyl-glucose suggests that the glutamate side chain is near the third hydroxyl group off glucose.

Surely, considering the application to glucose sensor constituent, the decrease in the reactivity toward allose and 3-*O*-methyl-glucose was not effective, because they were not existed in blood [40]. However, the decrease in the reactivity toward maltose was remarkable. Maltose can be introduced to peritoneal dialysis patients in the form of icodextrin-based dialysis solution (Extraneal: Baxter Healthcare SA, Castlebar, Ireland), thus increasing chances of overestimation of blood glucose concentration by sensors

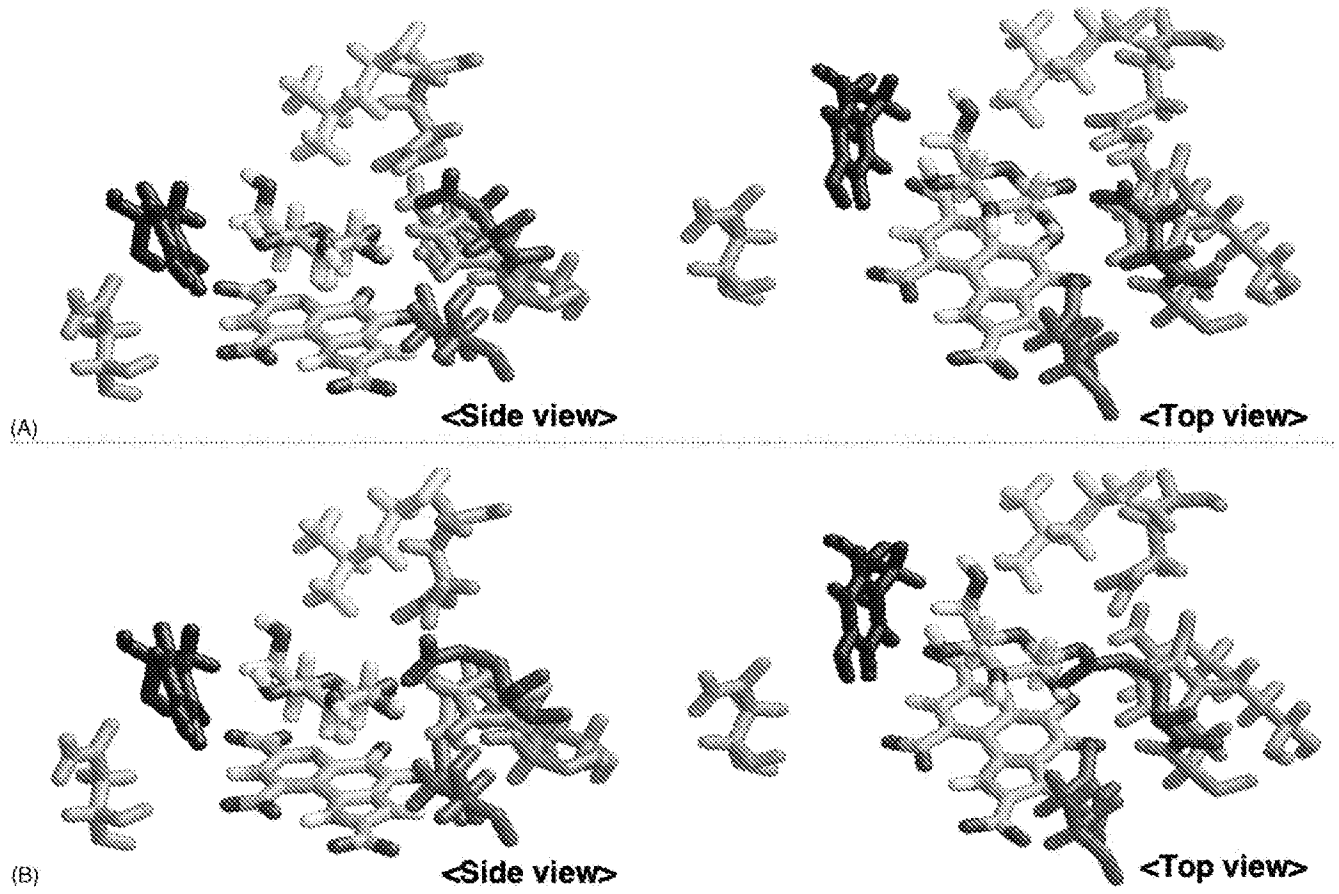

Fig. 4. Predicted structure of the active sites of wild-type (A) and Asp167Glu (B) PQQGDH-Bs. The residues shown are: Asp167 (red), Glu167 (red), Gln100 (magenta), His168 (green), Gln192 (violet), Leu193 (yellow), Tyr367 (blue), Asn452 (cyan), PQQ (gray and red), glucose (gray and red) and Ca^{2+} ion (yellow sphere).

based on enzyme less specific to glucose [41]. Therefore, our achievement in this study is useful for the development of ideal glucose sensor.

In order to investigate the effect of the replacement of Asp167 to Glu, we did three-dimensional structure prediction using molecular simulation software. In our constructed oxidized PQQGDH-B–glucose complex model (Fig. 4A), Asp167 (wild-type) makes hydrogen bond to glucose molecule at second hydroxyl group (2.86 Å). On the other hand, in Asp167Glu–glucose complex model (Fig. 4B), Glu167 made hydrogen bonds to second (2.22 Å) and third (3.02 Å) hydroxyl group of glucose without changing the environment in active site. Due to the formation of new hydrogen bond between Glu167 and third hydroxyl group, Asp167Glu demonstrated lower reactivity toward allose and 3-*O*-methyl-glucose. Asp167Glu consistently showed greatly reduced activities toward lactose and maltose (Table 4). The predicted models of Asp167Glu, although we expected that the decrease in activity toward lactose and maltose may therefore result from the recognition Tyr367 the only residue in proximity to the 4th OH of glucose and the non-reducing end of disaccharides, such alteration was not observed.

There appears to be a number of altered interactions responsible for the various changes in specificity profiles. Among them, we focused on a new orientation of substrate in the active site, the altered interactions between the substrate and surrounding residues may lead to a distorted substrate molecule in a different conformation than in the wild-type enzyme. In predicted model of Asp167Glu, Glu167 makes a hydrogen bond to third hydroxyl group of substrate. Therefore, it has a possibility the difference in substrate binding manner. Due to this binding manner, the distortion of substrate might be occurred and would affect the orientation of the non-reducing end of disaccharides, resulting in differences in the specificity between α-linked and β-linked disaccharides.

Especially, with cellobiose, another disaccharide with β1–4 linkage, the Asp167Glu was found to have over two-fold greater activity than wild-type (Table 4). These results suggest that the substrate recognition site of the non-reducing residue in Asp167Glu changed to suitable for cellobiose binding.

PQQGDH-B has attracted much attention as an alternative component to glucose oxidase (GOD) for glucose enzyme sensors. The superiority of PQQGDH-B resides in its higher

catalytic efficiency (PQQGDH-B: $154\,s^{-1}\,mM^{-1}$ versus GOD: $1.5\,s^{-1}\,mM^{-1}$) and its independence from dissolved oxygen for the oxidation of glucose. In the view of catalytic efficiency and substrate specificity profile, although membrane bound type glucose dehydrogenase (PQQGDH-A) was superior to PQQGDH-B (PQQGDH-B: $154\,s^{-1}\,mM^{-1}$ versus PQQGDH-A: $177\,s^{-1}\,mM^{-1}$), PQQGDH-B has great advantages on the recombinant production and the purification. These are important for the fabrication of glucose sensor constituents. Moreover, the substrate specificity profile of our mutant PQQGDH-Bs have achieved to the ideal levels. The Asp167Glu and Asp167Glu/Asn452Thr mutant enzymes created in this study showed narrower substrate specificity profiles than the wild-type enzyme, making them attractive candidates as glucose sensor constituents. The catalytic efficiencies for glucose of Asp167Glu ($31\,s^{-1}\,mM^{-1}$) and Asp167Glu/Asn452Thr ($25\,s^{-1}\,mM^{-1}$) remain considerably greater than that of GOD ($1.5\,s^{-1}\,mM^{-1}$). We recently achieved a drastic increase in thermal stability of PQQGDH-B by carrying out a Ser415Cys substitution, which resulted in the introduction of a disulfide bond in the dimer interface [33]. By combining the Asp167Glu or Asp167Glu/Asn452Thr mutations with the Ser415Cys mutation, we expect to create a thermostable and glucose-specific PQQGDH.

5. Conclusion

His168Cys and His168Gln showed greatly decreased k_{cat} and increased K_m values for glucose, helping to confirm His168's important catalytic role that had been proposed based on the enzyme's 3-D structure. The neighboring residues Lys166, Asp167, and Gln169 were then selected for mutagenesis because of their potential interactions with glucose. While mutants of Lys166 and Gln169 showed only minor changes in substrate specificity profiles, those of Asp167 mutants were greatly affected. Of the numerous Asp167 mutants characterized, Asp167Glu showed the best substrate specificity profile while retaining most of its catalytic activity for glucose. The relative activities for allose, 3-*O*-methyl-glucose, and maltose were particularly decreased. The combination Asp167Glu with Asn452Thr, a previously reported mutant with improved specificity, produced a cumulative effect on substrate specificity while retaining high catalytic efficiency. Therefore, newly created Asp167Glu and Asp167Glu/Asn452Thr mutants are attractive alternatives to glucose oxidase in the field of self-monitoring glucose sensors.

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